The Plant Journal (2003) **35**, 660–664 doi: 10.1046/j.1365-313X.2003.01836.x

TECHNICAL ADVANCE

Nuclear translocation of the photoreceptor phytochrome B is necessary for its biological function in seedling photomorphogenesis

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Received 7 May 2003; accepted 13 June 2003.
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Summary

The phytochrome (phy) family of sensory photoreceptors (phyA to phyE in *Arabidopsis*) enables plants to optimize their growth and development under natural light environments. Subcellular localization studies have shown that the photoreceptor molecule is induced to translocate from cytosol to nucleus by light, but direct evidence of the functional relevance of this translocation has been lacking. Here, using a glucocorticoid receptor-based fusion protein system, we demonstrate that both photoactivation and nuclear translocation combined are necessary and sufficient for the biological function of phyB. Conversely, neither artificial nuclear translocation of non-photoactivated phyB nor artificial retention of photoactivated phyB in the cytosol provides detectable biological activity. Together these data indicate that signal transfer from photoactivated phyB to its primary signaling partner(s) is localized in the nucleus, and conversely suggest the absence of a cytosolic pathway from photoactivated phyB to light-responsive genes.

Keywords: glucocorticoid receptor, light signaling, nuclear translocation, photoreceptor, phytochrome, subcellular distribution.

Introduction

Light is essential for growth, development, and survival of plants. For this reason, plants are equipped with an intricate set of sensory photoreceptors that have evolved to track this vital commodity (Kendrick and Kronenberg, 1994; Smith, 2000). The best-characterized photoreceptors are the phytochromes (phy), which are encoded by a small gene family designated *PHYA* to *PHYE* in *Arabidopsis* (Clack *et al.*, 1994; Sharrock and Quail, 1989). The phy molecule is a soluble chromoprotein comprising a bilin chromophore attached to a polypeptide, and is capable of light-induced interconversion between two conformers, designated Pr and Pfr. Red light converts Pr to Pfr, and far-red light converts Pfr back to Pr. Light-induced Pfr formation triggers an intracellular signaling process that induces alterations in expression of the genes that drive photomorphogenesis (Quail, 2002; Smith, 2000). Although the phy signaling mechanism is still elusive, recent discoveries have significantly changed prevailing views of the molecular, cellular, and biochemical processes involved in the signaling pathway (Quail, 2002). Importantly, although phys were previously considered to be constitutively cytoplasmically localized soluble proteins, recent studies have shown that phyA and phyB translocate into the nucleus in response to light (Hisada *et al.*, 2000; Kircher *et al.*, 2002; Nagy and Schafer, 2002; Sakamoto and Nagatani, 1996). However, direct evidence that this translocation is functionally necessary for phy signaling has yet to be provided. Here, using a glucocorticoid receptor (GR)-based fusion protein system to control nucleo-cytoplasmic partitioning independent of the light signal, we demonstrate that nuclear import of phyB is necessary for its biological function.

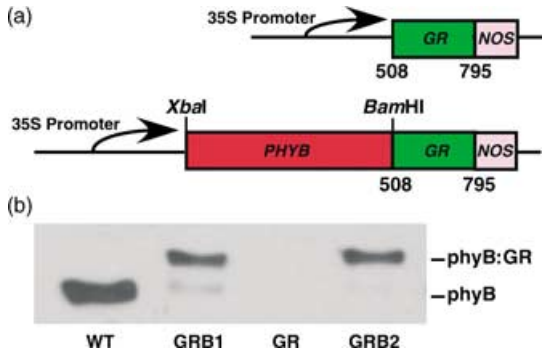

Figure 1. Expression of phyB:GR fusion protein in transgenic *phyB* mutant.
(a) Schematic representation of the constructs used for generating transgenic plants. GR, glucocorticoid receptor; phyB, phytochrome B.
(b) Immunoblots showing the level of expression of phyB:GR fusion protein compared to the wild-type (WT) control. Twenty micrograms of total protein was separated on an 8% SDS–PAGE gel, and probed with phyB-specific monoclonal antibodies.

Results and discussion

The GR-based fusion protein system has been used widely to control nucleo-cytoplasmic partitioning of nuclear proteins (Lloyd *et al.*, 1994; Samach *et al.*, 2000; Schena *et al.*, 1991; Wagner *et al.*, 1999). We made a translational fusion construct where phyB is fused in-frame to GR (Figure 1), transformed this construct into a *phyB*-null *Arabidopsis* mutant (Reed *et al.*, 1993), and selected multiple independent single insertion lines (only two are shown here). Western blotting showed that the phyB:GR fusion protein is expressed at the expected size in these transgenic lines, albeit at a somewhat lower level compared to native phyB in wild-type seedlings (Figure 1b).

We examined the capacity of the phyB:GR fusion protein to complement the phyB-null phenotype in response to continuous red light (Rc), with or without the steroid

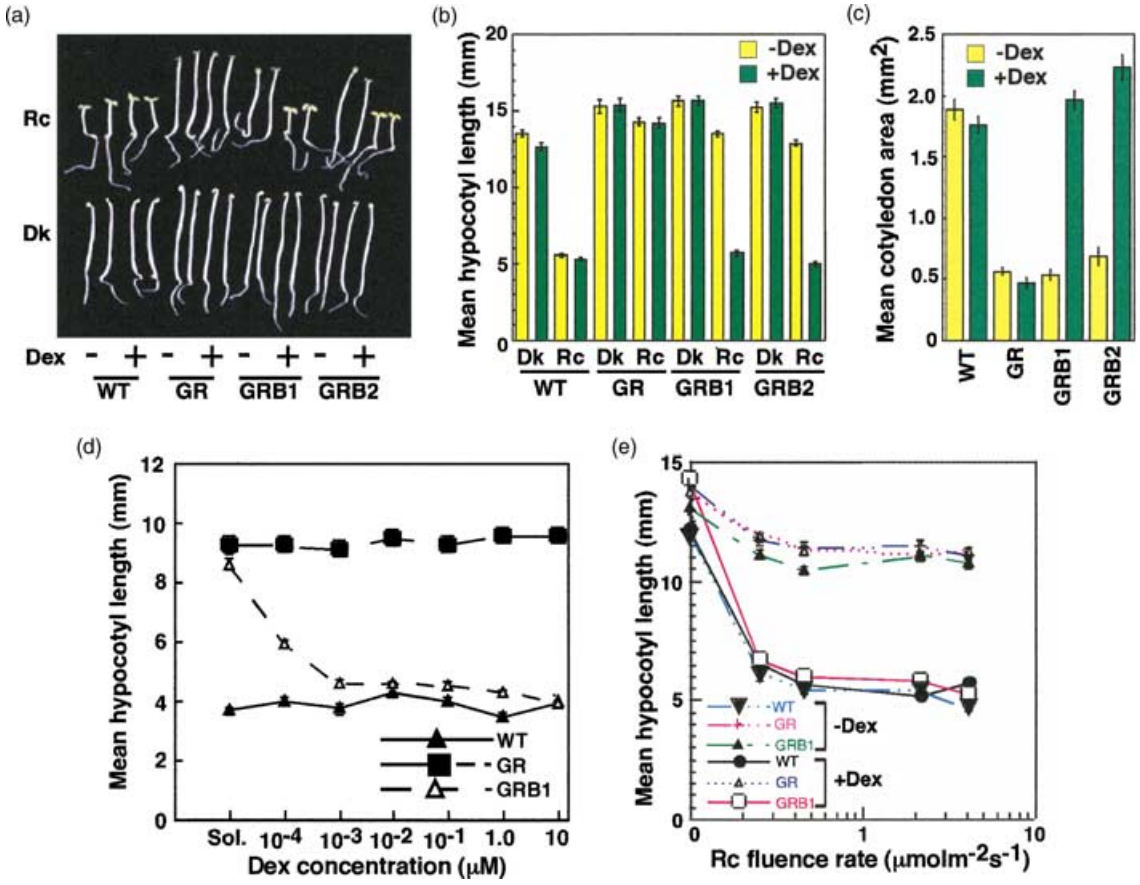

Figure 2. Transgenic complementation of the *phyB*-null mutant phenotype with the phyB:GR fusion protein in a Dex-dependent manner.
(a) Visual phenotypes of the WT and transgenic *phyB* mutant transformed with vector only (GR) or *PHYB:GR* construct (GRB1 and GRB2, two independent lines) grown for 4 days in the dark (Dk) or Rc (4 μmol m^{-2} sec^{-1}) in the presence (+) or absence (–) of 0.1 μM Dex.
(b, c) Bargraphs showing hypocotyl lengths and cotyledon areas of the lines described in (a), respectively. For cotyledon area, all the lines were grown under Rc (13 μmol m^{-2} sec^{-1}) for 4 days.
(d) Concentration curve for Dex-dependent mutant rescue. Seeds were plated on GM minus Suc plates containing different concentrations of Dex or on the solvent ethanol as a mock control. The seedlings were grown under Rc (13 μmol m^{-2} sec^{-1}) for 3 days. WT, GR and GRB1 are described in (a). Sol., ethanol solvent.
(e) Fluence-rate–response curves showing hypocotyl lengths of the seedlings for WT, vector control (GR), and one of the transgenic *phyB* lines (GRB1), grown for 3 days under various fluence rates of Rc in the presence (+) or absence (–) of 0.1 μM Dex. SEMs are shown as error bars.

dexamethasone (Dex). The phyB:GR-expressing transgenic seedlings displayed complete rescue of the seedling de-etiolation phenotypes of the *phyB* mutant, including suppression of hypocotyl length and expansion of cotyledons in the presence of Dex under Rc (Figure 2). In contrast, Rc without Dex, or Dex in the dark, did not rescue any aspect of these morphological phenotypes. Figure 2(a) shows the visible phenotype of the rescued mutant compared to the wild type, and the differences between the various genotypes and treatments are quantified for hypocotyl length and cotyledon area in Figure 2(b,c). Figure 2(d) shows that mutant rescue is quantitatively dependent on Dex concentration, saturating at 10^{-3} μM.

Fluence-rate–response curves show that in the absence of Dex, hypocotyl lengths of GRB1 seedlings (expressing the phyB:GR fusion protein) respond minimally to Rc, and are similar to those of the GR-control seedlings (expressing only the GR moiety) under all the fluence rates tested (Figure 2e). This result indicates the absence of any detectable phyB activity in these seedlings in the absence of Dex, even at the highest Rc fluence rates, implying highly stringent denial of phyB access to its signaling partners, despite maximal photoactivation. In contrast, addition of Dex under the same conditions results in a dramatic suppression of hypocotyl elongation for the GRB1, but not GR-control, seedlings under all fluence rates tested (Figure 2e). This response of the GRB1 seedlings is quantitatively indistinguishable from the wild type, indicating that the phyB:GR fusion protein is functionally just as active as the native photoreceptor molecule is upon Dex treatment. We were not able to test the flowering-time phenotype of the rescued phyB mutant because of the technical difficulty of growing the plants in the presence of Dex to maturity. However, we have observed that the phyB:GR transgenic plants flowered early, similar to the GR-control plants, relative to the wild type in the absence of Dex under greenhouse conditions (data not shown). Taken together, the above results indicate that the phyB:GR fusion protein is fully biologically active in transgenic *Arabidopsis* plants, complementing the *phyB*-null mutant in a Dex-dependent manner under Rc.

To determine whether the phyB:GR fusion protein does indeed translocate into the nucleus in a Dex-dependent manner, we examined its distribution between nuclear and cytoplasmic fractions by cell fractionation analysis (Figure 3; Table 1). The DNA content of the nuclear fraction was consistent between treatments within an experiment (Table 1), and the presence of the α-subunit of RNA polymerase was used to verify that our procedure provides quantitative and equivalent recovery of nuclear protein components in the designated nuclear fractions (Figure 3b). The fractionation data show that the phyB:GR fusion protein is enriched in the nuclear fraction in the presence of Dex, both under Rc and in the dark, compared to the minus Dex control (Figure 3a; Table 1). Although we

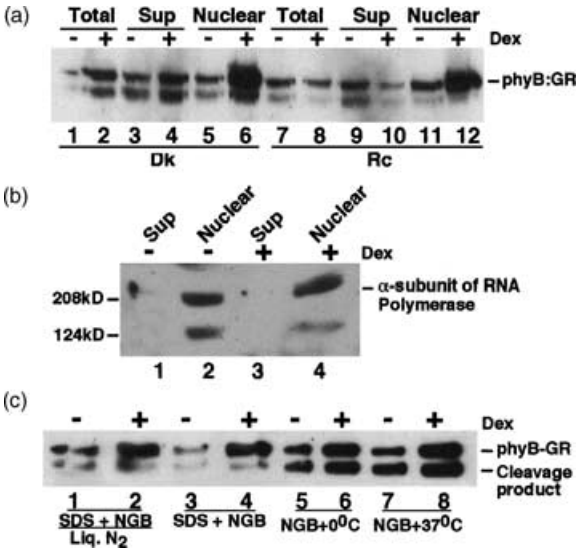

Figure 3. Dex-dependent enrichment of phyB:GR in nuclear fractions.
(a) Western blot showing distribution of phyB:GR fusion protein between nuclear and cytosolic fractions. Nuclei were isolated from GRB1 seedlings grown for 4 days in the dark (Dk) or under Rc in the presence (+) or absence (−) of 1 μM Dex. Forty micrograms protein from total extracts (Total) and first supernatants (Sup), and 20 μg protein from nuclear fractions (Nuclear) from +Dex and −Dex seedlings were separated on an 8% SDS–PAGE gel, blotted and probed with phyB-specific monoclonal antibodies.
(b) Recovery and verification of loading of equivalent amounts of nuclear-derived proteins in control and Dex-treated nuclear fractions. Quantitative recovery and loading of equivalent amounts of nuclear-derived proteins were further verified by probing immunoblots with antibodies against the α-subunit of RNA polymerase. Lanes 1 and 2 (mock treatment without Dex) correspond to the same extracts as lanes 9 and 11 in (a). Lanes 3 and 4 (+Dex treatment) correspond to the same extracts as lanes 10 and 12 in (a). Sixty micrograms of total supernatant protein was loaded in lanes 1 and 3, and 15 μg total nuclear fraction protein was loaded in lanes 2 and 4.
(c) Rapid post-homogenization cleavage of phyB:GR. Lanes 1–8 contain 60 μl of total homogenate after removal of cellular debris. Seedlings in lanes 1 and 2 were ground in liquid nitrogen and then extracted directly in boiling 2× SDS-Laemmli buffer (SDS)/2× NGB (SDS + NGB/Liq. N_2). Seedlings in lanes 3 and 4 were extracted directly in boiling 2× SDS-Laemmli buffer/2× NGB (SDS + NGB), seedlings in lanes 5 and 6 were extracted in 2× NGB on ice and after 1 min brought to 1× SDS-Laemmli buffer (NGB + 0°C), seedlings in lanes 7 and 8 were extracted in 2× NGB on ice, incubated 1 h at 37°C and brought to 1× SDS-Laemmli buffer (NGB + 37°C). The data indicate a rapid cleavage event, even at 0°C, in the standard NGB containing all readily available classes of protease inhibitors.

consistently detected an additional band of lower molecular weight than the full-length fusion protein (Figure 3a), the evidence indicates that this is an *in vitro* proteolytic degradation product (Figure 3c). The appearance of this band could be largely prevented by homogenizing the tissue directly in the presence of SDS-Laemmli buffer, but not by using a broad collection of commonly used protease inhibitors (Figure 3c). We conclude that this band corresponds to a rapid post-homogenization cleavage product.

Data from six independent replicates show that the phyB:GR fusion protein is enriched in the nuclear fraction in the presence of Dex by approximately eightfold under Rc and approximately sixfold in the dark (Table 1). This level

Table 1 Quantitation of phyB:GR enrichment in nuclear fractions

Treatments	PhyB:GR in the nuclear fraction	Dex-induced fold-enrichment in the nuclear fraction
Dk (−Dex)	3.7 ± 0.9	
Dk (+Dex)	19.2 ± 4.4	6.4 ± 1.4
Rc (−Dex)	4.8 ± 1.1	
Rc (+Dex)	34.2 ± 7.4	8.0 ± 1.9

Fold-enrichment of phyB:GR in the nuclear fractions was assessed by comparing signals on immunoblots of dilution series of first supernatants and nuclear fractions from +Dex and −Dex seedlings. The distribution of phyB:GR molecules between these two fractions was established visually from these dilution curves (expressed as percentage phyB:GR in the nuclear fraction). The Dex-dependent enrichment in the nuclear fraction was then expressed as a ratio of the percentage nuclear phyB:GR in +Dex versus −Dex fractions (Dex-induced fold-enrichment). The mean percentage of phyB:GR in the respective nuclear fractions, and the fold-enrichment values ±standard error for six independent determinations for dark (Dk)- and Rc-grown seedlings are presented. The variability in DNA content between nuclear fractions from the different treatments was limited on average to ±10% of the mean within an experiment (data not shown).

corresponds to approximately 34% of the total detectable phyB:GR in the cell in Rc and approximately 19% in the dark. The Rc-induced enrichment of phyB:GR here is slightly higher than that of native phyB or phyB:GFP fusion protein in nuclear fractions from tobacco as observed by others (Nagy and Schafer, 2002). The appearance of phyB:GR in the nuclear fraction in the dark in the presence of Dex is presumably because of the presence of nuclear localization signals (NLSs) in the GR moiety (Picard and Yamamoto, 1987). The apparently greater Dex-induced nuclear enrichment of phyB:GR in Rc-grown seedlings than in dark-grown seedlings (Figure 3a; Table 1) might be caused by the additive effect of the GR NLS and the natural NLS responsible for native phyB translocation in Rc. Regardless, the Dex-induced translocation in dark-grown seedlings suggests the absence of an active retention mechanism for the Pr form of phyB in the cytosol as postulated by Fankhauser *et al*. (1999).

Taken together, the data presented here suggest that: (i) nuclear translocation is necessary but not sufficient for phyB biological function, as the Pr form appears to have no functional activity in the nucleus in inducing seedling de-etiolation; (ii) light-induced Pfr formation is necessary but not sufficient for phyB biological function, as the Pfr form appears to have no functional activity in seedling de-etiolation when retained in the cytoplasm; but (iii) nuclear translocation and Pfr formation are together both necessary and sufficient for phyB biological function. These findings provide compelling evidence that the critical molecular events constituting signal transfer from photoactivated phyB to its primary signaling partner(s) are localized in the nucleus and occur obligatorily in the Pfr form. This conclusion is consistent with a current model proposing that the primary mechanism of phy signaling involves direct interaction with a transcription factor PIF3 bound to the promoters of photoresponsive target genes (Martinez-Garcia *et al.*, 2000; Quail, 2002).

Experimental procedures

Construction of the vector and analysis of transgenic plants

For expressing the phyB:GR fusion protein, the open-reading frame of the phyB cDNA was amplified using PFU Turbo polymerase (Stratagene, La Jolla, CA, USA) with forward (5′-GAtctagaATGGTTTCCGGAGTCGGGGGTAG-3′) and reverse (5′-CAggatccGCATATGGCATCATCAGCATCATG-3′) primers, which included *Xba*I and *Bam*HI restriction sites for cloning. The resulting fragment was cloned into the GR vector (kindly provided by Dr Alan M. Lloyd, University of Texas at Austin, USA) using *Xba*I-*Bam*HI restriction sites, and sequenced. This construct was electroporated into GV3101 (MP90) *Agrobacterium* and used for transformation of *phyB* mutant (Reed *et al.*, 1993) by the floral dip method (Clough and Bent, 1998). Transgenic seeds were plated on growth medium without sucrose (GM minus Suc) plates containing 50 μg ml^{-1} of kanamycin. The resistant seedlings were transplanted to soil and grown in the greenhouse. For Western blots, 200 μg FW of seedlings grown for 3 days in the dark and irradiated for 24 h under Rc 20 μmol m^{-2} sec^{-1} were homogenized in 500 μl extraction buffer (100 mM MOPS (pH 7.6), 10% glycerol, 40 mM beta-mercaptoethanol, 20 mM iodoacetamide, 2 mM PMSF, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin and 1 μg ml^{-1} leupeptin). The extracts were cleared by centrifugation and 20 μg of total protein was separated on an 8% SDS–PAGE gel, blotted and probed with phyB-specific B1–B7 monoclonal antibodies (Hirschfeld *et al.*, 1998).

Seedling growth and measurements

Sterilization of seeds and plating on GM minus Suc and hypocotyl length measurements were performed as described by Huq and Quail (2002). The light sources are described by Wagner *et al.* (1991). Fluence rates of red light were measured by a spectroradiometer (model LI-1800, LiCor, NE).

Preparation of nuclear extracts and immunoblotting

Four-day-old GRB1 seedlings were grown in the dark or under Rc in the presence of 1 μM Dex or equivalent volume of solvent (ethanol, mock treatment). Nuclei were isolated as described by Lissemore and Quail (1988) with the following modifications: 1 g of tissue was homogenized in 3 ml of nuclear grinding buffer (NGB) consisting of 100 mM MOPS (pH 7.6), 5% Dextran T-40, 2.5% Ficoll, 0.25 M sucrose, 40 mM beta-mercaptoethanol, 10 mM $MgCl_2$, 20 mM iodoacetamide, 2 mM PMSF, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin, and 1 μg ml^{-1} leupeptin. The extract was then passed over two layers of miracloth, 70 and 30 μm exclusion filters. Organellar membranes were lysed in the presence of 0.5% Triton X-100, the filtrate was then centrifuged at 3700 ***g***, and the pellets were washed twice in the presence of 0.1% Triton X-100. The nuclear

fraction pellet was finally dissolved in 300 μl of NGB and sonicated twice for 15 sec. Forty micrograms protein of total unfractionated extracts and first supernatants from Dex- and mock-treated seedlings, and 20 μg protein from nuclear fractions from Dex- and mock-treated seedlings were separated on an 8% SDS–PAGE gel, blotted and probed with phyB monoclonal antibodies. To ensure equal loading of nuclear protein, 15 μg of nuclear fractions were probed with polyclonal anti-RNA polymerase antibody (Covance, Denver, PA, USA).

Acknowledgements

We thank Christina Lanzatella for excellent technical assistance. This work was supported by grants from the Torrey Mesa Research Institute, San Diego, DOE Basic Energy Sciences number DE-FG01–87ER11742 and US Department of Agriculture Current Research Information Service number 5115-21000-070–00D.

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